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(71) Applicant: KOURION THERAPEUTICS GMBH  
[DE/DE]; Himmelgeister Strasse 248, 40225 Düsseldorf  
(DE).

(72) Inventor: WERNET, Peter; Im Winkel 1, 40627 Düsseldorf  
(DE).

(73) Agents: MEYERS, Hans-Wilhelm et al.; Postfach 10 22  
41, 50462 Köln (DE).

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(54) Title: HUMAN CORD BLOOD DERIVED UNRESTRICTED SOMATIC STEM CELLS (USSC)

(57) Abstract: A composition in human cord and placental blood which comprises unrestricted somatic stem cells is described here which can be amplified in vitro to large quantities sufficient for medical applications as regenerative medicines. Initiation and maintenance as well as ex vivo expansion protocols of such stem cells from cord blood is described. Furthermore, it is shown that from these cells employing varying differentiation induction protocols distinct lineage progenitors for hematopoiesis and endothel, as well as mesenchymal progenitors for muscle bone, cartilage and fat as well as neural progenitors can be cultured and expanded for use in regenerative medicine.

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# INTERNATIONAL SEARCH REPORT

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**A. CLASSIFICATION OF SUBJECT MATTER**  
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**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ERICES A ET AL: "Mesenchymal progenitor cells in human umbilical cord blood." BRITISH JOURNAL OF HAEMATOLOGY, vol. 109, no. 1, April 2000 (2000-04), pages 235-242, XP002205332 ISSN: 0007-1048 the whole document</p> <p style="text-align: center;">--- -/--</p>	1-11

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Teyssier, B

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In ☐ International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ALFONSO Z Z C &amp; FRASER J K: "Osteoblast precursor cells are found in the low-density fraction of umbilical cord blood." BLOOD, vol. 94, no. 10 Suppl. 1 Part 2, 15 November 1999 (1999-11-15), page 161b XP002231452</p> <p>Forty-first Annual Meeting of the American Society of Hematology; New Orleans, Louisiana, USA; 3-7 December 1999 ISSN: 0006-4971 Abstract 3897</p> <p>---</p>	1-11
A	<p>GUTIERREZ-RODRIGUEZ M ET AL: "Characterization of the adherent cells developed in Dexter-type long-term cultures from human umbilical cord blood." STEM CELLS, vol. 18, no. 1, January 2000 (2000-01), pages 46-52, XP002231492 ISSN: 1066-5099</p> <p>---</p>	
A	<p>SIRCHIA G &amp; REBULLA P: "Placental/umbilical cord blood transplantation." HAEMATOLOGICA, vol. 84, no. 8, August 1999 (1999-08), pages 738-747, XP002226904 ISSN: 0390-6078</p> <p>---</p>	
P, A	<p>SANCHEZ-RAMOS J R ET AL: "Expression of neural markers in human umbilical cord blood." EXPERIMENTAL NEUROLOGY, vol. 171, no. 1, September 2001 (2001-09), pages 109-115, XP002226905 ISSN: 0014-4886</p> <p>---</p>	
P, X	<p>GOODWIN H S ET AL: "Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers." BIOLOGY OF BLOOD AND MARROW TRANSPLANTATION, vol. 7, no. 11, November 2001 (2001-11), pages 581-588, XP008012375 ISSN: 1083-8791 the whole document</p> <p>---</p> <p style="text-align: center;">-/--</p>	1-11

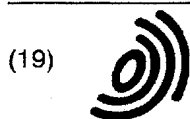
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>WERNET P ET AL: "Detection of unrestricted multipotential stem cells in human cord blood."  BLOOD,  vol. 98, no. 11 Part 1,  16 November 2001 (2001-11-16), page 550a  XP002226906  43rd Annual Meeting of the American Society of Hematology; Orlando, Florida, USA; 7-11 December 2001  ISSN: 0006-4971</p> <p>-----</p>	



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(72) Inventors:

- **HONMOU, Osamu**  
Chuo-ku, Sapporo-shi, Hokkaido 064-0801 (JP)
- **HASHI, Kazuo**  
Sapporo-shi, Hokkaido 064-0942 (JP)
- **UEDE, Teiji**  
Sapporo-shi, Hokkaido 004-0882 (JP)

(71) Applicant: **Renomedix Institute Inc.**  
**Sapporo-shi, Hokkaido 060-0061 (JP)**

(74) Representative: **VOSSIUS & PARTNER**  
**Siebertstrasse 4**  
**81675 München (DE)**

(54) **CELL FRACTION CONTAINING CELLS CAPABLE OF DIFFERENTIATING INTO NEURAL CELLS**

(57) Demyelinated axons were remyelinated in the demyelinated rat model by collecting bone marrow cells from mouse bone marrow and transplanting the mononuclear cell fraction separated from these bone marrow cells.

**EP 1 302 534 A1**

## Description

### Technical Field

[0001] The present invention relates to cells derived from bone marrow cells, cord blood cells, or embryonic hepatic tissues that can differentiate into neural cells, and cell fractions containing such cells. It is expected that these cells and cell fractions can be used to treat neurological diseases, particularly in autologous transplantation therapy.

### Background Art

[0002] Transplantation of oligodendrocytes (i.e., oligodendroglia) (Archer D. R., et al., 1994. Exp. Neurol. 125:268-77; Blakemore W. F., Crang A. J., 1988. Dev. Neurosci. 10:1-11; Gumpel M., et al. 1987. Ann. New York Acad. Sci. 495:71-85) or myelin-forming cells, such as Schwann cells (Blakemore W. F., 1977. Nature 266: 68-9; Blakemore W. F., Crang A. J., 1988. Dev. Neurosci. 10:1-11; Honmou O. et al., 1996. J. Neurosci. 16: 3199-208), or olfactory ensheathing cells (Franklin R. J. et al., 1996. Glia 17:217-24; Imaizumi T. et al., 1998. J. Neurosci. 18(16):6176-6185; Kato T. et al., 2000. Glia 30:209-218), can elicit remyelination in animal models and recovery of electrophysiological function (Utzschneider D. A. et al., 1994. Proc. Natl. Acad. Sci. USA. 91: 53-7; Honmou O. et al., 1996. J. Neurosci. 16: 3199-208). It is possible to prepare such cells from patients or other persons for cell therapy. However, this method is considerably problematic because tissue material must be collected from either the brain or nerves.

[0003] Neural progenitor cells or stem cells derived from brain have the ability to self-renewal and differentiate into various lineages of neurons and glia cells (Gage F.H. et al., 1995. Proc. Natl. Acad. Sci. USA. 92: 11879-83; Lois C., Alvarez-Buylla A., 1993. Proc. Natl. Acad. Sci. USA. 90:2074-7; Morshead C. M. et al., 1994. Neuron 13:1071-82; Reynolds B. A., Weiss S., 1992. Science 255:1707-10). By transplantation into newborn mouse brain, human neural stem cells collected from fetal tissues differentiate into neurons and astrocytes (Chalmers-Redman R.M. et al., 1997. Neurosci. 76: 1121-8; Moyer M. P. et al., 1997. Transplant. Proc. 29: 2040-1; Svendsen C.N. et al., 1997. Exp. Neurol. 148: 135-46), and myelinate the axons (Flax J. D. et al., 1998. Nat. Biotechnol. 16:1033-9). Remyelination and recovery of impulse conduction upon transplantation of neural progenitor (stem) cells derived from adult human brain into demyelinated rodent spinal cord have been reported (Akiyama Y. et al., 2001. Exp. Neurol.).

[0004] These studies have evoked great interest due to the indicated possibility of the application of the above-mentioned cells to regenerative strategy of neurological diseases (Akiyama Y. et al., 2001. Exp. Neurol.; Chalmers-Redman R. M. et al., 1997. Neurosci. 76: 1121-8; Moyer M. P. et al., 1997. Transplant. Proc. 29:

2040-1; Svendsen C. N. et al., 1997. Exp. Neurol. 148: 135-46; Yandava B. D. et al., 1999. Proc. Natl. Acad. Sci. USA. 96:7029-34). However, in order to establish cell transplantation therapy (including autologous transplantation) using these cells, still problems, such as establishment of harvest method and requirement of cell expansion using trophic factors, remain to be solved.

[0005] According to the recent studies, neural stem cells were revealed to be able to differentiate or transform into hematopoietic cells *in vivo*, suggesting that neural progenitor (stem) cells are not restricted to the neural cell lineage (Bjornson C. R. et al., 1999. Science 283:534-7). Furthermore, bone marrow stromal cells are reported to differentiate into astrocytes by the injection into the lateral ventricles of neonatal mice (Kopen G.C. et al., Proc. Natl. Acad. Sci. USA. 96:10711-6), and into neurons *in vitro* when cultured under appropriate cell culture conditions (Woodbury D. et al., 2000. J. Neurosci. Res. 61:364-70).

### Disclosure of the Invention

[0006] The present inventors have previously isolated and cultured neural stem cells from adult human brain, and established some cell lines. By studying their functions, the inventors newly discovered that the neural stem cells have pluripotency and the ability to self-renewal. Specifically, single-cell expansion of neural progenitor (stem) cells obtained from adult human brain was conducted to establish cell lines; the established cells were then subjected to *in vitro* clonal analysis. The result showed that the cell lines had pluripotency (namely, differentiation into neuron, astroglia (or astrocyte), and oligodendroglia (i.e., oligodendrocyte)) and the ability to self-renewal (namely, proliferation potency). Thus, these cells were confirmed to possess the characteristics of neural stem cell.

[0007] Transplantation of these cells indeed resulted in very favorable graft survival, migration, and differentiation in cerebral ischemic model rats or injury model rats. Furthermore, transplantation of the cells was found to result in functional myelin sheath formation in spinal cord demyelination model rats. Thus, such transplantation allows remyelination of the demyelinated axon and restoration of the neural function in the rat spinal cord demyelination model. Effectiveness of such transplantation therapy using these cells was confirmed by histological, electrophysiological, and behavior studies.

[0008] Judging from the above-described findings, transplantation of cultured neural stem cells, which have been isolated from a small amount of neural tissue collected from the cerebrum of a patient, into the lesion of the spinal cord of the patient seems to be a widely applicable in autotransplantation therapy.

[0009] However, while not causing neurologic deficits, collecting tissues containing neural stem cells from cerebrum is relatively invasive. Thus, considering the need for establishing therapeutic methods for various compli-

cated diseases in the nervous system today, it is crucial to establish a safer and simpler method for autotransplantation therapy.

**[0010]** Thus, an objective of the present invention is to provide cellular material that is useful in the treatment of neurological diseases, and which can be prepared safely and readily. Another objective of the present invention is to provide a method for treating neurological diseases, preferably a method for autotransplantation therapy, using the cellular material.

**[0011]** In view of the existing state as described above, to establish donor cells the present inventors focused on the technique of collecting bone marrow cells from bone marrow, a simpler technique as compared to the collection of neural stem cells and routinely used in today's medical practice. First, they collected bone marrow cells from mouse bone marrow, isolated mononuclear cell fraction, and then transplanted this fraction as donor cells into spinal cord demyelination model rats. Surprisingly, it was discovered that the demyelinated axon gets remyelination by the treatment. Hence, the present inventors newly revealed that the mononuclear cell fraction prepared from bone marrow cells have the ability to differentiate into neural cells. The present inventors also discovered that cell fractions containing mesodermal stem cells, mesenchymal stem cell, stromal cells, and AC133-positive cells, that were isolated from the mononuclear cell fraction had the ability to differentiate into neural cells. Besides bone marrow cells, these cell fractions can also be prepared from cord blood cells. Furthermore, AC133-positive cells can be prepared from embryonic hepatic tissues.

**[0012]** Thus, the present invention provides cell fractions containing cells capable of differentiating into neural cells, which are isolated from bone marrow cells, cord blood cells, and embryonic hepatic tissues.

**[0013]** In another embodiment, such cell fractions contain mesenchymal stem cells having the following character: SH2(+), SH3(+), SH4(+), CD29(+), CD44(+), CD14(-), CD34(-), and CD45(-).

**[0014]** In another embodiment, such cell fractions contain stromal cells having the following characteristics: Lin(-), Sca-1(+), CD10(+), CD11D(+), CD44(+), CD45(+), CD71(+), CD90(+), CD105(+), CDW123(+), CD127(+), CD164(+), fibronectin (+), ALPH(+), and collagenase-1(+).

**[0015]** In another embodiment, such cell fractions contain cells having the character AC133(+).

**[0016]** In addition, the present invention provides cells capable of differentiating into neural cells, which are contained in the above-mentioned cell fraction.

**[0017]** Furthermore, the present invention provides compositions for treating neurological disease, which contain the above-mentioned mononuclear cell fractions or the above-mentioned cells. According to a preferred embodiment of the present invention, the neurological disease is selected from the group consisting of: central and peripheral demyelinating diseases; central

and peripheral degenerative diseases; cerebral apoplexy; brain tumor; dysfunction of higher function of the brain; psychiatric diseases; dementia; epilepsy; traumatic neurological diseases; and infarction of spinal cord diseases.

**[0018]** Furthermore, the present invention provides therapeutic methods for neurological diseases, which comprises transplanting of the above-mentioned mononuclear cell fractions, cells, or compositions. In preferred embodiments, the donor cells are derived from a recipient.

**[0019]** The present invention provides mononuclear cell fractions isolated from bone marrow cells, cord blood cells, or embryonic hepatic tissues, wherein the fractions contain cells capable of differentiating into neural cells. It is unclear whether the differentiation of cells contained in the cell fractions provided by the present invention into neural cells is caused by the transformation of so-called hematopoietic cells into neural cells, or, alternatively, by the differentiation of immature cells capable of differentiating into neural cells that are comprised in bone marrow cells, etc. However, the majority of the cells differentiating into neural cells are assumed to be stem or precursor cells, namely, cells having the self-renewal ability and pluripotency. Alternatively, the cells differentiating into neural cells may be stem or precursor cells which have differentiated to some extent into endoderm or mesoderm.

**[0020]** Cells in a cell fraction of the present invention do not have to be proliferated with any trophic factors (then again they can proliferate in the presence of trophic factors). Thus, these cells are simple and practical from the standpoint of the development of autotransplantation technique for the diseases in the nervous system, and are very beneficial in medical industry. In general, a cell fraction of the present invention is derived from vertebrate, preferably from mammal (for example, mouse, rat, human, etc.).

**[0021]** A cell fraction of the present invention can be prepared by subjecting bone marrow cells or cord blood cells collected from vertebrate to density-gradient centrifugation at 2,000 rpm in a solution for a sufficient time ensuring separation depending on specific gravity, and then recovering the cell fraction with a certain specific gravity within the range of 1.07 to 1.1 g/ml. Herein, the phrase "a sufficient time ensuring separation depending on specific gravity" refers to a time sufficient for the cells to shift to a position in the solution according to their specific gravity, which is typically about 10 to 30 minutes. The specific gravity of the cell fraction to be recovered is within the range of 1.07 to 1.08 g/ml (for example, 1.077 g/ml). Solutions, such as Ficoll solution and Percoll solution, can be used for the density-gradient centrifugation, but is not limited thereto.

**[0022]** Specifically, first, bone marrow (5 to 10  $\mu$ l) collected from a vertebrate is combined with a solution (2 ml L-15 plus 3 ml Ficoll), and then centrifuged at 2,000 rpm for 15 minutes to isolate a mononuclear cell fraction

(approx. 1 ml). The mononuclear cell fraction is combined with culture solution (2ml NPBm) to wash the cells, and then the cells are again centrifuged at 2,000 rpm for 15 minutes. Then, the precipitated cells are recovered after the removal of the supernatant. Besides femur, sources to obtain a cell fraction of the present invention include sternum, and ilium constituting the pelvis. Any other bone can serve as a source so long as it is large enough. A cell fraction of the present invention can also be prepared from bone marrows and cord blood stored in bone marrow bank or cord blood bank.

**[0023]** Another embodiment of cell fractions of the present invention includes a mononuclear cell fraction isolated and purified from bone marrow cells or cord blood cells, which contains mesodermal (mesenchymal) stem cells capable of differentiating into neural cells. The term "mesodermal (mesenchymal) stem cell" refers to cells that can copy (divide and proliferate) cells with the same potential as themselves and that are capable of differentiating into any type of cells constituting mesodermal (mesenchymal) tissues. Mesodermal (mesenchymal) cells indicate cells constituting tissues that are embryologically categorized to the mesoderms, including blood cells. The mesodermal (mesenchymal) stem cell includes, for example, cells characterized by SH2(+), SH3(+), SH4(+), CD29(+), CD44(+), CD14(-), CD34(-), and CD45(-). A cell fraction containing mesodermal (mesenchymal) stem cells can be obtained, for example, by selecting cells having a cell surface marker, such as SH2, as described above from the above-mentioned cell fraction obtained by centrifuging bone marrow cells or cord blood cells (the cell fraction according to claim 2).

**[0024]** Furthermore, a cell fraction containing mesodermal (mesenchymal) stem cells capable of differentiating into neural cells can be prepared by subjecting bone marrow cells or cord blood cells collected from vertebrate to density-gradient centrifugation at 900 G in a solution for a sufficient time ensuring separation depending on specific gravity, and then recovering the cell fraction with a certain specific gravity within the range of 1.07 to 1.1 g/ml. Herein, "a sufficient time ensuring separation depending on specific gravity" refers to a time sufficient for the cells to shift to a specific position corresponding to their specific gravity in the solution for density-gradient centrifugation, which is typically about 10 to 30 minutes. The specific gravity of a cell fraction to be recovered varies depending on the type of animal (for example, human, rat, and mouse) from which the cells have been derived. A solution to be used for density-gradient centrifugation includes Ficoll solution and Percoll solution, but is not limited thereto.

**[0025]** Specifically, first, bone marrow (25 ml) or cord blood collected from vertebrate is combined with an equal volume of PBS solution, and then centrifuge at 900 G for 10 minutes. Precipitated cells are mixed with PBS and then are recovered (cell density = approx.  $4 \times 10^7$  cells/ml) to remove blood components. Then, a 5-ml

aliquot thereof is combined with Percoll solution (1.073 g/ml), and centrifuged at 900 G for 30 minutes to extract a mononuclear cell fraction. The extracted mononuclear cell fraction is combined with a culture solution (DMEM, 10% FBS, 1% antibiotic-antimycotic solution) to wash the cells, and is centrifuged at 2,000 rpm for 15 minutes. Finally, the supernatant is removed, precipitated cells are recovered and cultured at 37°C under 5% CO<sub>2</sub> atmosphere.

**[0026]** Another embodiment of a cell fraction of the present invention is a fraction of mononuclear cells isolated from bone marrow cells or cord blood cells, which contains stromal cells capable of differentiating into neural cells. Examples of stromal cell include cells characterized by Lin(-), Sca-1(+), CD10(+), CD11D(+), CD44(+), CD45(+), CD71(+), CD90(+), CD105(+), CDW123(+), CD127(+), CD164(+), fibronectin(+), ALPH(+), and collagenase-1(+). A cell fraction containing stromal cells can be prepared, for example, by selecting cells having a cell surface marker, such as Lin as described above, from the above-mentioned cell fraction obtained by centrifuging bone marrow cells or cord blood cells (the cell fraction according to claim 2).

**[0027]** Furthermore, such a cell fraction can be prepared by subjecting bone marrow cells or cord blood cells collected from vertebrate to density-gradient centrifugation at 800 G in a solution for a sufficient time ensuring separation depending on specific gravity, and then recovering the cell fraction with a certain specific gravity within the range of 1.07 to 1.1 g/ml. Herein, "a sufficient time ensuring separation depending on the specific gravity" indicates a time sufficient for the cells to shift to a specific position corresponding to their specific gravity in the solution for density-gradient centrifugation, which is typically about 10 to 30 minutes. The specific gravity of a cell fraction to be recovered is preferably within the range of 1.07 to 1.08 g/ml (for example, 1.077 g/ml). A solution to be used for density-gradient centrifugation includes Ficoll solution and Percoll solution, but is not limited thereto.

**[0028]** Specifically, first, bone marrow or cord blood collected from vertebrate is combined with an equal volume of a solution (PBS, 2% BSA, 0.6% sodium citrate, and 1% penicillin-streptomycin). A 5-ml aliquot thereof is combined with Ficoll + Paque solution (1.077 g/ml) and centrifuged at 800 G for 20 minutes to obtain a mononuclear cell fraction. The mononuclear cell fraction is combined with a culture solution (Alfa MEM, 12.5% FBS, 12.5% horse serum, 0.2% i-inositol, 20 mM folic acid, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 1 μM hydrocortisone, 1% antibiotic-antimycotic solution) to wash the cells, and then are centrifuged at 2,000 rpm for 15 minutes. The supernatant is removed after centrifugation. The precipitated cells are collected and then cultured at 37°C under 5% CO<sub>2</sub> atmosphere.

**[0029]** Another embodiment of a cell fraction of the present invention is a mononuclear cell fraction containing cells characterized by AC133(+) capable of differen-

tiating into neural cells, which is isolated from bone marrow cells, cord blood cells, or embryonic hepatic tissues. Such a cell fraction can be obtained, for example, by selecting cells having a cell surface marker including the above-mentioned AC133(+) from the cell fraction obtained, as described above, by centrifuging bone marrow cells or cord blood cells (the cell fraction according to claim 2).

**[0030]** Furthermore, the cell fraction can be obtained by subjecting embryonic hepatic tissues collected from vertebrate to density-gradient centrifugation at 2,000 rpm in a solution for a sufficient time ensuring separation depending on specific gravity, recovering a cell fraction within the range of a specific gravity of 1.07 to 1.1 g/ml, and then recovering cells with the characteristic of AC133(+) from the cell fraction. Herein, "a sufficient time ensuring separation depending on specific gravity" indicates a time sufficient for the cells to shift to a specific position corresponding to their specific gravity in the solution for density-gradient centrifugation, which is typically about 10 to 30 minutes. The solution to be used for density-gradient centrifugation includes Ficoll solution and Percoll solution, but is not limited thereto.

**[0031]** Specifically, first, liver tissue collected from vertebrate is washed in L-15 solution, and then treated enzymatically in an L-15 solution containing 0.01% DNaseI, 0.25% trypsin, and 0.1% collagenase at 37°C for 30 minutes. Then, the tissue is dispersed into single cells by pipetting. The single-dispersed embryonic hepatic cells are centrifuged by the same procedure as described for the preparation of the mononuclear cell fraction from femur in Example 1(1). The cells thus obtained are washed, and then AC133(+) cells are collected from the washed cells using an AC133 antibody. Thus, cells capable of differentiating into neural cells can be prepared from embryonic hepatic tissues. The antibody-based recovery of AC133(+) cells can be achieved using magnetic beads or a cell sorter (FACS, etc.).

**[0032]** Transplantation of any of these cell fractions containing mesodermal stem cells, mesenchymal stem cells, stromal cells, or AC133-positive cells into demyelinated spinal cord can lead to efficient remyelination of the demyelinated region. In particular, the above-mentioned cell fraction containing mesenchymal stem cells can engraft favorably and differentiate into cells in neural lineages including glia when transplanted into a cerebral infarction model.

**[0033]** The present invention also provides cells capable of differentiating into neural cells, which are contained in the above-mentioned cell fraction. These cells include, for example, neural stem cells, mesodermal stem cells, mesenchymal stem cells, stromal cells, and AC133-positive cells which are contained in the above-mentioned cell fraction, but are not limited thereto so long as they can differentiate into neural cells.

**[0034]** The present invention also provides compositions for treating neurological diseases, which comprise a cell fraction or cells of the present invention. It is pos-

sible to transplant the cell fractions or cells of the present invention without modification. However, in order to improve the efficiency of therapy, they may be transplanted as compositions combined with various additives or introduced with genes. The preparation of compositions of the present invention may comprise, for example, (1) addition of a substance that improves the proliferation rate of cells included in a cell fraction of the present invention or enhances the differentiation of the cells into neural cells, or introducing a gene having the same effect; (2) addition of a substance that improves the viability of cells in a cell fraction of the present invention in damaged neural tissues, or introducing a gene having the same effect; (3) addition of a substance that inhibits adverse effects of damaged neural tissue on the cells in a cell fraction of the present invention, or introducing a gene having the same effect; (4) addition of a substance that prolongs the lifetime of donor cells, or introducing a gene having the same effect; (5) addition of a substance that modulates the cell cycle, or introducing a gene having the same effect; (6) addition of a substance to suppress the immunoreaction or inflammation, or introducing a gene having the same effect; (7) addition of a substance that enhances the energy metabolism, or introducing a gene having the same effect; (8) addition of a substance that improves the migration of donor cells in host tissues, or introducing a gene having the same effect; and (9) addition of a substance that improves blood flow (including inductions of angiogenesis), or introducing a gene having the same effect, but is not limited thereto.

**[0035]** A cell fraction, cell, and composition of the present invention can be used for treating neurological diseases. Target neurological diseases for the therapy include, for example, central and peripheral demyelinating diseases; central and peripheral degenerative diseases; cerebral apoplexy (including cerebral infarction, cerebral hemorrhage, and subarachnoid hemorrhage); cerebral tumor; disorders of higher brain function including dementia; psychiatric diseases; epilepsy, traumatic neurological diseases (including head injury, cerebral contusion, and spinal cord injury); and infarction of spinal cord, but are not limited thereto.

**[0036]** According to the present invention, cells derived from bone marrow cells of a recipient can be transplanted as donor cells (autotransplantation therapy). This has the following advantages: (1) low risk of rejection for the transplantation; and (2) no difficulty in using immunosuppressant. When autotransplantation therapy is arduous, then cells derived from other person or nonhuman animal may be used. Cells frozen for storage are also usable. The donor cells may be derived from cord blood.

**[0037]** Bone marrow can be collected, for example, by anesthetizing (by local or systemic anesthesia) an animal (including human) that serves as a source, put a needle into the sternum or iliac of the animal, and aspirating the bone marrow with a syringe. On the other

hand, it is an established technique to collect cord blood at birth by putting needle directly into the umbilical cord, and aspirating the blood from the umbilical cord using syringe, and to store the blood.

[0038] Transplantation of cells into a patient can be performed, for example, by first filling a syringe with cells to be transplanted. Herein, the cells are suspended in an artificial cerebrospinal fluid or physiological saline. Then, the damaged neural tissue is exposed by surgery, and, with a needle, directly injecting the cells into the lesion. Due to high migrating potential of cells contained in a cell fraction of the present invention, they can migrate in the neural tissues. Hence, cells can be transplanted into a region adjacent to the lesion. Moreover, injection of the cells into the cerebrospinal fluid is also expected to be efficacious. In the case of the injection of the cells into the cerebrospinal fluid, the cells can be injected into a patient by typical lumbar puncture, without surgical operation only under local anesthetization. Thus, the patient can be treated in patient's sickroom (not in an operation room), which makes the method preferable. Alternatively, intravenous injection of the cells is also expected to be effective. Thus, transplantation can be carried out by a procedure based on typical blood transfusion, which is advantageous in that the treatment can be performed in patient's sickroom.

[0039] Furthermore, due to their high migrating potential, cells in a cell fraction of the present invention can be used as a carrier (vector) for genes. For example, the cells are expected to be useful as a vector for gene therapy for various neurological diseases such as brain tumor.

#### Brief Description of the Drawings

#### [0040]

Fig. 1 shows optical light micrographs of transections of dorsal column of the spinal cord, which had been prepared at 1-mm intervals. (A), normal axon; and (C), damaged demyelinated axon. Patterns of dorsal column observed at higher magnification are shown in (B) for normal axon and in (D) for demyelinated axon. Scale bars: 250  $\mu$ m in (A) and (C); 10  $\mu$ m in (B) and (D).

Fig. 2 shows microphotographs demonstrating the remyelination of rat spinal cord (A), after transplantation of adult mouse bone marrow cells; and (C), after transplantation of Schwann cells. Photomicrographs demonstrating remyelinated axon observed at higher magnification are shown in (B), after transplantation of bone marrow cells; and (D), after transplantation of Schwann cells. Scale bars: 250  $\mu$ m in (A) and (C); 10  $\mu$ m in (B) and (D).

Fig. 3 shows an electron micrograph of remyelinated axon after the transplantation of bone marrow into the dorsal columns. The tissue was treated with substrate X-Gal to detect transplanted bone mar-

row cells containing the (3-galactosidase gene in the damaged tissues (the reaction product is indicated with arrow). When observed at higher magnification, basal lamina was found around the fibers (wedge-shaped mark; scale bar 1  $\mu$ m). The presence of large cytoplasmic and nuclear regions and basal lamina in the transplanted cells indicates peripheral myelination.

#### 10 Best Mode for Carrying out the Invention

[0041] The present invention will be described in more detail below with reference to examples based on specific experiments.

#### 15 [Example 1] Preparation of bone marrow cells and Schwann cells

##### (1) Bone marrow mononuclear cells

[0042] Mouse bone marrow cells (10  $\mu$ l) were obtained from the femur of adult LacZ (a structural gene of  $\beta$ -galactosidase) transgenic mice (The Jackson Laboratory, Maine, USA). The collected sample was diluted in L-15 medium (2 ml) containing 3 ml Ficoll, and centrifuged at 2,000 rpm for 15 minutes. Cells were collected from the mononuclear cell fraction, and suspended in 2 ml serum-free medium (NPM: Neural Progenitor cell Maintenance Medium). Following centrifugation (2,000 rpm, 15 min), the supernatant was removed, and precipitated cells were collected and re-suspended in NPM.

##### (2) Schwann cells

[0043] Primary Schwann cell cultures were established from the sciatic nerve of neonatal mouse (P1-3) according to the method of Honmou et al. (J. Neurosci., 16(10): 3199-3208, 1996). Specifically, cells were released from sciatic nerve by enzymatic and mechanical treatment.  $8 \times 10^5$  cells per plate were plated onto 100-mm<sup>2</sup> poly (L-lysine)-coated tissue culture plates and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum.

#### [Example 2] Experimental animal preparation and transplantation

##### 50 (1) Preparation of demyelinated rat model

[0044] Experiments were performed on 12 week old Wistar rats. A localized demyelinated lesion was created in the dorsal columns using X-ray irradiation and ethidium bromide injection (EB-X treatment). Specifically, rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) i.p., and a surface dose of 40 Grays of X-ray was irradiated using Softex M-150 WZ radio-

therapy machine (100 kV, 1.15 mA, SSD 20 cm, dose rate 200 cGy/min) on the spinal cord caudal to the tenth thoracic spine level (T-10) through a 2 x 4 cm opening in a lead shield (4 mm thick). Three days after X-ray irradiation, rats were anesthetized as above, and aseptic laminectomy of the eleventh thoracic spine (T-11) was conducted. A demyelinating lesion was generated by the direct injection of ethidium bromide (EB) into the dorsal columns via a glass micropipette whose end was drawn. 0.5  $\mu$ l saline containing 0.3 mg/ml EB was injected at the depths of 0.7 and 0.4 mm.

(2) Transplantation of stem or progenitor cells that can differentiate or transform into the neural lineages

[0045] 3 days after the EB injection, 1  $\mu$ l of the cell suspension ( $1 \times 10^4$  cells/ $\mu$ l), which was obtained in Example 1, was injected into the middle of the EB-X-induced lesion. Transplanted rats were immunosuppressed with cyclosporin A (10 mg/kg/day).

#### [Example 3] Histological examination

[0046] Rats were deeply anesthetized by the administration of sodium pentobarbital (60 mg/kg, i.p.), and perfused through the heart cannula, first, with phosphate-buffer solution (PBS) and then with a fixative containing 2% glutaraldehyde and 2% paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4. Following *in situ* fixation for 10 minutes, the spinal cord was carefully excised, cut into 1 mm segments and kept in fresh fixative. The tissue was washed several times in Sorensen's phosphate buffer, post-fixed in 1% OsO<sub>4</sub> for 2 hours at 25°C, dehydrated by elevating the concentration of the ethanol solution, passed through propylene oxide and embedded in EPON. Then, the tissue was cut into sections (1  $\mu$ m), counterstained with 0.5% methylene blue and 0.5% azure II in 0.5% borax, and examined under light microscope (Zeiss: Axioskop FS). Ultrathin sections were counterstained with uranyl and lead salts, and examined with JEOL JEM1200EX electron microscope (JEOL, Ltd., Japan) at 60 kV.

[0047] A 50 x 50  $\mu$ m standardized region in the central core of the dorsal columns in the spinal cords near the site wherein the cells were initially injected was used for morphometric analysis. The numbers of remyelinated axons and cell bodies associated with the axons were counted within this region; and the density to square millimeters was calculated. Furthermore, the diameters of the axons and cell bodies, the number of cells with multilobular nuclei, and cells showing myelination were examined in the same standardized region. Measurements and counts were obtained from five sections per rat, and five rats (n=5) were analyzed for each experimental condition. All variances represent standard error ( $\pm$  SEM).

[0048] The dorsal column in the spinal cord mostly consists of myelinated axons (Fig. 1A, B). The prolifer-

ation of endogenous glial cells was inhibited by the irradiation of X-ray to the dorsal columns of the lumbar spinal cord. Further, by the administration of a nucleic acid chelator, ethidium bromide, glial cells such as oligodendrocytes were found damaged and local demyelination occurred. Such lesions generated according to this procedure are characterized by almost complete loss of endogenous glial cells (astrocytes and oligodendrocytes) and preservation of axons (Fig. 1C). Examination of the lesion with a light microscope under a higher magnification revealed that congested areas consisting of demyelinated axons are appositioned closely to one another separated by areas wherein the debris of myelin exist and wherein macrophages exist (Fig. 1D). The lesion occupied nearly the entire dorso-ventral extent of the dorsal columns 5 to 7 mm longitudinally. Almost none of the endogenous invasion of Schwann cells or astrocytes occurs till the sixth to eighth week. With the start of invasion, these glial cells begin to invade into the lesion from peripheral borders. Thus, a demyelinated and glial-free environment is present for at least 6 weeks *in vivo*.

[0049] Three weeks after transplantation of LacZ transgenic mouse bone marrow cells (BM) into the central region of the lesion in immunosuppressed and demyelinated rat models, extensive remyelination of the demyelinated axons was observed (Figs. 2A and 2B). Remyelination was observed across the entire coronal dimension of the dorsal columns and throughout the antero-posterior extent of the lesion. Figure 2C and 2D show the pattern of remyelination observed after transplantation of allogeneic Schwann cells (SC) into the EB-X lesion model. It is remarkable that large nuclear and cytoplasmic regions were found around the remyelinated axons, a characteristic of peripheral myelin, by both BM and SC transplantations.

#### [Example 4] Detection of $\beta$ -galactosidase reaction products *in vivo*

[0050] Three weeks after transplantation,  $\beta$ -galactosidase expressing myelin-forming cells were detected *in vivo*. Spinal cords were collected and fixed in 0.5% glutaraldehyde in phosphate-buffer for 1 h. Sections (100  $\mu$ m) were cut with a vibratome and  $\beta$ -galactosidase expressing myelin-forming cells were detected by incubating the sections at 37°C overnight with X-Gal (substrate which reacts with  $\beta$ -galactosidase to develop color) at a final concentration of 1 mg/ml in X-Gal developer (35 mM K<sub>3</sub>Fe(CN)<sub>6</sub>/35 mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O/2 mM MgCl<sub>2</sub> in phosphate-buffered saline) to form blue color within the cell. Sections were then fixed for an additional 3 h in 3.6% (vol/vol) glutaraldehyde in phosphate-buffer (0.14 M), and were examined with light microscope for the presence of blue reaction product ( $\beta$ -galactosidase reaction product). Prior to embedding in EPON, the tissue was treated with 1% OsO<sub>4</sub>, dehydrated in a graded series of ethanol, and soaked in propylene oxide for a short

period. Ultrathin sections were then examined under an electron microscope without further treatment.

**[0051]** Under the electron microscope, most of the myelin-forming cells derived from donor cells retained the basal membrane (Fig. 3: wedge-shaped mark). Additionally, the myelin-forming cells had relatively large nucleus and cytoplasm, which suggests the formation of a peripheral nervous system-type myelin sheath.

**[0052]** It was confirmed that almost no endogenous remyelination by oligodendrocytes or Schwann cells occurs for at least six weeks in the lesion model used in the present experiment. Furthermore, the donor cells that contained the reporter gene LacZ, i.e., X-Gal-positive cells, were observed to form myelin at the electron microscopic level (Fig. 3: arrow).

**[0053]** Differentiation into neurons and glial cells could be observed following the transplantation of bone marrow cells into the EB-X lesions, but not by SC transplantation. Five percent of lacZ-positive cells (transplanted bone marrow cells) in the EB-X lesions showed NSE (Neuron Specific Enolase)-immunoreactivity and 3.9% showed GFAP (Glial Fibrillary Acidic Protein)-immunoreactivity, indicating that some of the bone marrow cells can differentiate into neurons or glial cells, respectively, *in vivo*.

**[0054]** Furthermore, employing antibodies, the present inventors isolated mesenchymal stem cells with the characteristic of cell markers SH2(+), SH3(+), CD29(+), CD44(+), CD14(-), CD34(-), and CD45(-) from the cell fraction obtained in Example 1 (1). Furthermore, they discovered that transplantation of the cells into the demyelinated regions of rat spinal cord results in more efficient remyelination. It was also revealed that the cells survived favorably and differentiated into neurons or neuronal cells and glia cells when transplanted into cerebral infarction model rats.

**[0055]** Further, the present inventors isolated stromal cells characterized by the cell surface markers Lin(-), Sca-1(+), CD10(+), CD11D(+), CD44(+), CD45(+), CD71(+), CD90(+), CD105(+), CDW123(+), CD127(+), CD164(+), fibronectin (+), ALPH(+), and collagenase-1 (+) from the cell fraction obtained in Example 1 (1). Transplantation of the cells into demyelinated regions of rat spinal cord also resulted in efficient remyelination.

**[0056]** Further, the present inventors isolated cells characterized by the cell surface marker AC133(+) from the cell fraction obtained in Example 1 (1). Transplantation of the cells into demyelinated regions of rat spinal cord also resulted in efficient remyelination.

**[0057]** In addition, the present inventors obtained a cell fraction containing AC133-positive cells capable of differentiating into neural cells from rat embryonic hepatic tissues by the following procedure. Specifically, first, liver tissues collected from rat fetuses were washed in L-15 solution, and then treated enzymatically in L-15 solution containing 0.01% DNaseI, 0.25% trypsin, and 0.1% collagenase at 37°C for 30 minutes. Then, the tissue was dispersed into single cells by pipetting several

times. The single-dispersed embryonic hepatic tissues were centrifuged as in Example 1(1) (preparation of mononuclear cell fraction from femur) to isolate a mononuclear cell fraction. The obtained mononuclear cell fraction was washed, and then, AC133(+) cells were recovered from the cell fraction using anti-AC133 antibody. The isolation of AC133-positive cells can be achieved using magnetic beads or a cell sorter (FACS or the like). Transplantation of the obtained AC133-positive cells into demyelinated regions of rat spinal cord also resulted in efficient remyelination.

#### Industrial Applicability

**[0058]** As described above, the present invention provides fractions of mononuclear cells isolated and purified by collecting bone marrow-derived bone marrow cells, cord blood-derived cells, or fetal liver-derived cells. Transplantation of such mononuclear cell fractions into a demyelination model animal was confirmed to result in remyelination of the demyelinated axon.

**[0059]** Cells for transplantation can be relatively easily isolated from a small quantity of bone marrow cell fluid aspirated from bone marrow, and can be prepared for transplantation in several tens of minutes after the cells are being collected. Thus, these cells can serve as useful and regenerable cellular material for autotransplantation in the treatment of demyelinating diseases.

**[0060]** This invention highlights the development of the autotransplantation technique to treat demyelinating diseases in the central nervous system. Furthermore, the use of the present invention in transplantation and regeneration therapy for more general and diffuse damage in the nervous system is envisaged. In other words, this invention sheds light on autotransplantation therapy against ischemic cerebral damage, traumatic cerebral injury, cerebral degenerating diseases, and metabolic neurological diseases in the central and peripheral nervous systems.

**[0061]** According to the present invention, cells in the hematopoietic system (bone marrow or cord blood) are used as donor cells. Thus, to treat neurological diseases, the cells may be transplanted into the vessels instead of directly transplanting them into neural tissues. Specifically, donor cells transplanted into a vessel can migrate to the neural tissues and thereby regenerate the neural tissues. Hence, the present invention is a breakthrough in developing a therapeutic method for noninvasive transplantation.

**[0062]** Furthermore, the present invention adds significantly to elucidate the mechanism underlying the differentiation of non-neural cells such as hematopoietic cells and mesenchymal cells into neural cells. When genes determining the differentiation are identified and analyzed, use of such genes will allow efficient transformation of a sufficient quantity of non-neural cells such as hematopoietic cells and mesenchymal cells in a living body to neural cells. Thus, the present invention is a

breakthrough in the field of "gene therapy" for inducing regeneration of neural tissues.

#### Claims

1. A mononuclear cell fraction containing cells capable of differentiating into neural cells, wherein said fraction is isolated from bone marrow cells or cord blood cells collected from vertebrate.
2. The cell fraction according to claim 1, which can be prepared by subjecting bone marrow cells or cord blood cells collected from vertebrate to density-gradient centrifugation at 2,000 rpm in a solution for a sufficient time ensuring separation depending on specific gravity, and then recovering the cell fraction within the range of a specific gravity of 1.07 to 1.1 g/ml.
3. A mononuclear cell fraction isolated from bone marrow cells or cord blood cells collected from vertebrate, wherein the fraction contains cells having the character SH2(+), SH3(+), SH4(+), CD29(+), CD44(+), CD14(-), CD34(-), and CD45(-), and are capable of differentiating into neural cells.
4. The cell fraction according to claim 3, which can be prepared by recovering cells having the character SH2(+), SH3(+), SH4(+), CD29(+), CD44(+), CD14(-), CD34(-), and CD45(-), from the cell fraction according to claim 2.
5. The cell fraction according to claim 3, which can be prepared by subjecting bone marrow cells or cord blood cells collected from vertebrate to density-gradient centrifugation at 900 G in a solution for a sufficient time ensuring separation depending on specific gravity, and then recovering the cell fraction within the range of a specific gravity of 1.07 to 1.1 g/ml.
6. A mononuclear cell fraction containing cells isolated from bone marrow cells or cord blood cells collected from vertebrate, wherein the fraction contains cells having the character Lin(-), Sca-1(+), CD10(+), CD11D(+), CD44(+), CD45(+), CD71(+), CD90(+), CD105(+), CDW123(+), CD127(+), CD164(+), fibronectin (+), ALPH(+), and collagenase-1(+), and are capable of differentiating into neural cells.
7. The cell fraction according to claim 6, which can be prepared by recovering cells having the character Lin(-), Sca-1(+), CD10(+), CD11D(+), CD44(+), CD45(+), CD71(+), CD90(+), CD105(+), CDW123(+), CD127(+), CD164(+), fibronectin (+), ALPH(+), and collagenase-1(+) from the cell fraction according to claim 2.
8. The cell fraction according to claim 6, which can be prepared by subjecting bone marrow cells or cord blood cells collected from vertebrate to density-gradient centrifugation at 800 G in a solution for a sufficient time ensuring separation depending on specific gravity, and then recovering the cell fraction within the range of a specific gravity of 1.07 to 1.1 g/ml.
9. A mononuclear cell fraction isolated from bone marrow cells, cord blood cells, or embryonic hepatic tissues collected from vertebrate, wherein the fraction contains cells **characterized by** AC133(+), and are capable of differentiating into neural cells.
10. The cell fraction according to claim 9, which can be prepared by subjecting bone marrow cells, cord blood cells, or embryonic hepatic tissues collected from vertebrate to density-gradient centrifugation at 2,000 rpm in a solution for a sufficient time ensuring separation depending on specific gravity, recovering a cell fraction within the range of a specific gravity of 1.07 to 1.1 g/ml, and then recovering cells **characterized by** AC133(+) from the cell fraction.
11. A cell capable of differentiating into neural cells, which is contained in the cell fraction according to any one of claims 1 to 10.
12. A composition for treating neurological diseases, which comprises the cell fraction according to any one of claims 1 to 10, or the cell according to claim 11.
13. The composition according to claim 12, wherein the neurological disease is selected from the group consisting of central and peripheral demyelinating diseases; central and peripheral degenerative diseases; cerebral apoplexy; brain tumor; dysfunction of higher function of the brain; psychiatric diseases; epilepsy; traumatic neurological diseases; and infarction of spinal cord.
14. A method for treating neurological diseases, which comprises transplanting, into a recipient, the cell fraction according to any one of claims 1 to 10, the cell according to claim 11, or the composition according to claim 12.
15. The therapeutic method according to claim 14, wherein the neurological disease is selected from the group consisting of central and peripheral demyelinating diseases; central and peripheral degenerative diseases; cerebral apoplexy; brain tumor; dysfunction of higher function of the brain; psychiatric diseases; epilepsy; traumatic neurological diseases; and infarction of spinal cord.

16. The therapeutic method according to claim 15, wherein the cells to be transplanted are derived from the recipient.

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FIG. 1

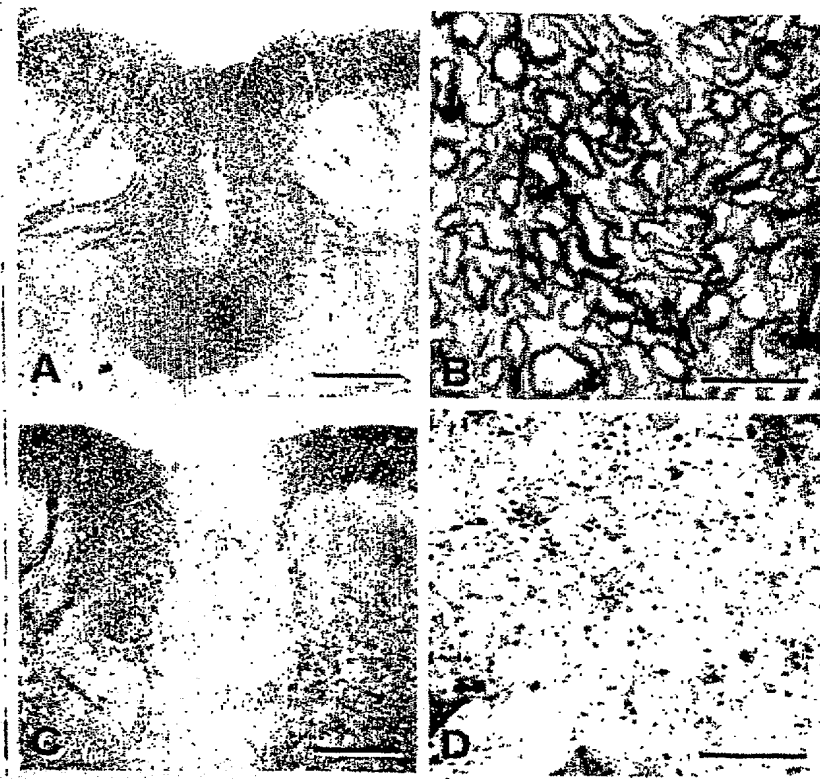


FIG 2

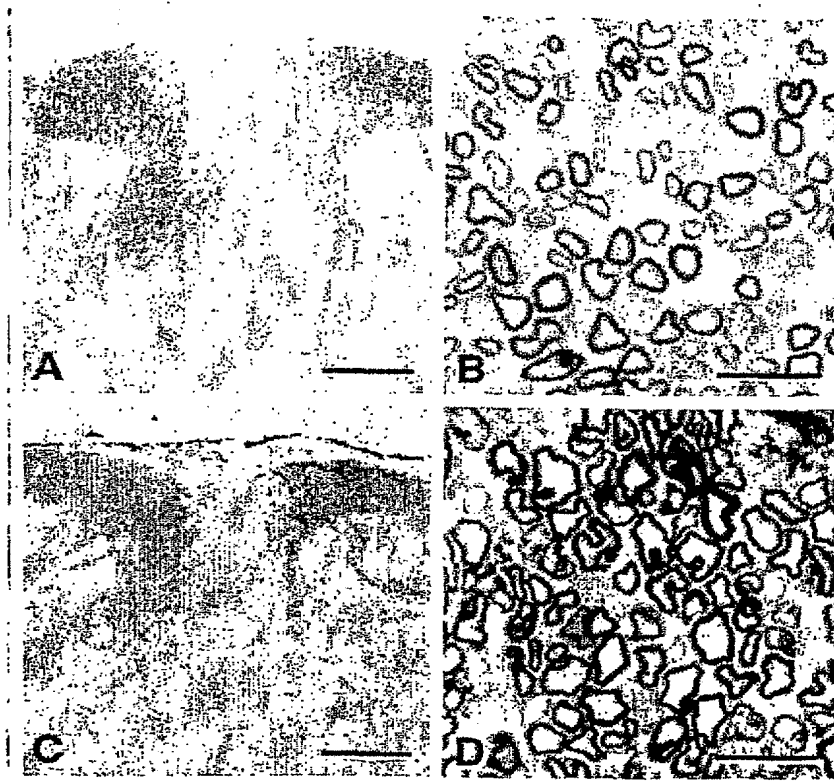


FIG. 3



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/05456

A. CLASSIFICATION OF SUBJECT MATTER Int.Cl <sup>7</sup> C12N 5/06, A61K 35/28, A61P 25/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int.Cl <sup>7</sup> C12N 5/06, A61K 35/28, A61P 25/00		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) MEDLINE (STN), WPI (DIALOG), BIOSIS (DIALOG)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SASAKI M. et al., "Transplantation of an Acutely Isolated Bone Marrow Fraction Repairs Demyelinated Adult Rat Spinal Cord Axons", GLIA, (2001), Vol.35, No.1, pages 26 to 34	1-13
P,X	WOODBURY D. et al., "Adult Rat and Human Bone Marrow Stromal Cells Differentiate Into Neurons", J. Neurosci. Res., August, 2000, Vol.61, pages 364 to 370	1-13
X	KOPENG C. et al., "Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains", Proc. Natl. Acad. Sci., U.S.A., (1999), Vol.96, pages 10711 to 10716	1-13
A	Honmou O. et al., "Restoration of Normal Conduction Properties in Demyelinated Spinal Cord Axons in the Adult Rat by Transplantation of Exogenous Schwann Cells", J. Neurosci., (1996), Vol.16, No.10, pages 3199 to 3208	1-13
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 04 September, 2001 (04.09.01)		Date of mailing of the international search report 18 September, 2001 (18.09.01)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/05456

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Bjornson C. R. R. et al., "Turning Brain into Blood: A Hematopoietic Fate Adopted by Adult Neural Stem Cells in Vivo", Science, (1999), Vol.283, No.5401, pages 534 to 537	1-13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/05456

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 14-16  
because they relate to subject matter not required to be searched by this Authority, namely:

These claims involve methods for treatment of the human body by therapy.

2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)